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TITLE: Ribozyme-Mediated Repair of Mutant p53 Transcripts in Breast Cancer Cells

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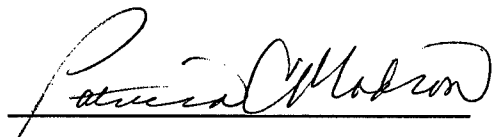
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13. ABSTRACT *(Maximum 200 words)*

This project explores the potential of a new and innovative approach to human gene therapy that may prove to be useful for the treatment or prevention of a range of genetic diseases including many types of cancer. We have previously demonstrated that a trans-splicing group I ribozyme can be employed to repair mutant transcripts in E. coli and mammalian cells. Ribozyme-mediated repair of mutant mRNAs associated with a range of human diseases is now experimentally tractable. In this study we have begun to explore the potential of this process for the repair of mutant transcripts implicated in the development and progression of breast cancer. Because mutation of the p53 gene is the most common genetic change seen in a wide variety of malignancies including breast cancer, we have initially focused upon the repair of mutant p53 transcripts. Toward this end, we have developed ribozymes that can react with mutant p53 RNAs in breast cancer cell lines and are now in the process of determining if the ribozymes can repair these mutant RNAs and revert the neoplastic phenotype of these cells.

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Bruce A. Sillman   9/11/98

PI - Signature   Date

RESEARCH GRANT

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1. Introduction
1.1 Overview
The overall goal of this project is to develop a new and innovative approach to human gene therapy that is based upon ribozyme-mediated repair of mutant mRNAs (Sullenger and Cech, 1994; Sullenger and Cech, 1995; Sullenger, 1995). This approach may be especially useful for the treatment of cancer because it should restore the regulated expression of oncogenes. Previously, we described how a trans-splicing ribozyme can be employed to repair mutant mRNAs in *Escherichia coli* (Sullenger and Cech, 1994). Subsequently we demonstrated that trans-splicing can also proceed in mammalians cells (Jones et al., 1996). Finally, we recently demonstrated that ribozymes could be employed to repair sickle ß-globin transcripts in erythrocyte precursors from patients with sickle cell disease (Lan et al., 1998).

Many mutant messenger RNAs from dominant and suppressor oncogenes have been identified that appear to be involved in tumorigenesis. Repair of any of these mRNAs may yield new insights into tumor development and treatment. However, we have initially focused upon the repair of mutant transcripts from the *p53* tumor suppressor gene. Much evidence indicates that loss of the p53 protein is associated with neoplastic transformation. Mutant *p53* transcripts are frequently found in a range of primary human tumors and tumor cell lines including mammary carcinomas (Nigro et al., 1989; Bartek et al., 1990; Malkin 1994). Thus we have sought to develop ribozymes that can repair the mutant p53 transcripts present in these tumor cells in hope of using such ribozymes to either revert the transformed phenotype of these cells or induce apoptosis. Toward this end the specific objectives of this research proposal have not changed and are:

1.) To determine which regions of mutant *p53* transcripts are accessible to trans-splicing ribozymes.
2.) To construct trans-splicing ribozymes that can repair mutant *p53* transcripts and test them in vitro.
3.) To evaluate ribozyme-mediated *p53* repair in tissue culture cells after transient transfection.
4.) To determine if the ribozyme can repair endogenous mutant *p53* transcripts in breast cancer cell lines harboring defective copies of the *p53* gene.
5.) To ascertain if repair of mutant *p53* transcripts renders breast cancer cells less tumorigenic.

1.2. Targeted trans-splicing by the *Tetrahymena* ribozyme.
1.2.a. RNA revision by ribozymes. During gene expression, the information contained in a given protein encoding gene is directly copied into the corresponding pre-messenger RNA by transcription. The information embedded in this RNA is not fixed however and can be modified by splicing (Moore et al., 1993; Ruby and Abelson, 1991; Guthrie, 1991; Green, 1991) or editing (Bass, 1993; Benne et al., 1986; Sollner-Webb,

1991) to remove, add or rewrite parts of the initial transcript. The self-splicing reaction of the group I intron ribozyme from *Tetrahymena thermophila* is perhaps the most thoroughly understood reaction that revises RNA. The intron performs two consecutive trans-esterification reactions to liberate itself and join flanking exons (Fig. 1A) (Cech, 1990).

In addition to performing a self-splicing reaction, a slightly shortened version of the group I ribozyme from *Tetrahymena* can splice an exon attached to its 3' end onto a targeted 5' exon RNA that is present in trans (Fig. 1B). In this reaction, the 5' exon is recognized by the group I ribozyme via base pairing through its 5' exon binding site. In the process of pairing, a U is positioned across from the guanosine present at the 5' end of the 5' exon binding site. Once positioned, the ribozyme catalyzes the cleavage of the substrate RNA at the reconstructed 5' splice site and then ligates its 3' exon onto the 5' exon cleavage product (Fig. 1B). Trans-splicing by group I ribozymes is extremely malleable because very few sequence requirements exist for the exons in this reaction. Virtually any U residue in a 5' exon can be targeted for splicing by altering the nucleotide composition of the 5' exon binding site on the ribozyme to make it complementary to the target site. Because no specific 3' exon sequences are required, such trans-splicing ribozymes can potentially be employed to splice virtually any 3' exon sequence onto a targeted U residue.

1.2.b. Directed RNA revision by trans-splicing. A trans-splicing ribozyme can be employed to revise the sequence of targeted RNAs. In the first example of this application, the group I ribozyme from *Tetrahymena* was re-engineered to repair truncated *lacZ* transcripts via targeted trans-splicing (Sullenger and Cech, 1994). In this system, the ribozyme's normal 3' exon was replaced with 200 nucleotides of *lacZ*. For trans-splicing to correct the defective *lacZ* messages, the ribozyme must recognize the truncated 5' *lacZ* RNA by base pairing, cleave off additional nucleotides, hold onto the 5' exon cleavage product by maintaining base pairing through the 5' exon-binding site of the ribozyme, and ligate the *lacZ* 3' exon sequence onto the cleaved 5' product to yield the correct translational reading frame (Fig. 2). It was shown that the ribozyme could faithfully accomplish such RNA revision both *in vitro* and in *Escherichia coli* (Sullenger and Cech, 1994).

After we had demonstrated that ribozymes could repair mutant transcripts in bacteria, we wanted to determine if such trans-splicing could repair mutant *lacZ* transcripts in the more therapeutically relevant setting of mammalian cells. To monitor targeted trans-splicing in transfected OST7-1 cells, we isolated total RNA and amplified the splicing products by reverse transcription and the polymerase chain reaction (RT/PCR). RT/PCR was performed using one primer specific for the targeted 5' *lacZ* sequence and the other primer specific for the *lacZ* 3' exon tag. Using these primers, an amplified fragment of expected size (200 base pairs) was generated from RNA isolated from the OST7-1 cells transfected with the active ribozyme containing

Figure 1. Self-Splicing and Targeted Trans-Splicing by the Tetrahymena Ribozyme

A. Self-Splicing of a Group I Intron
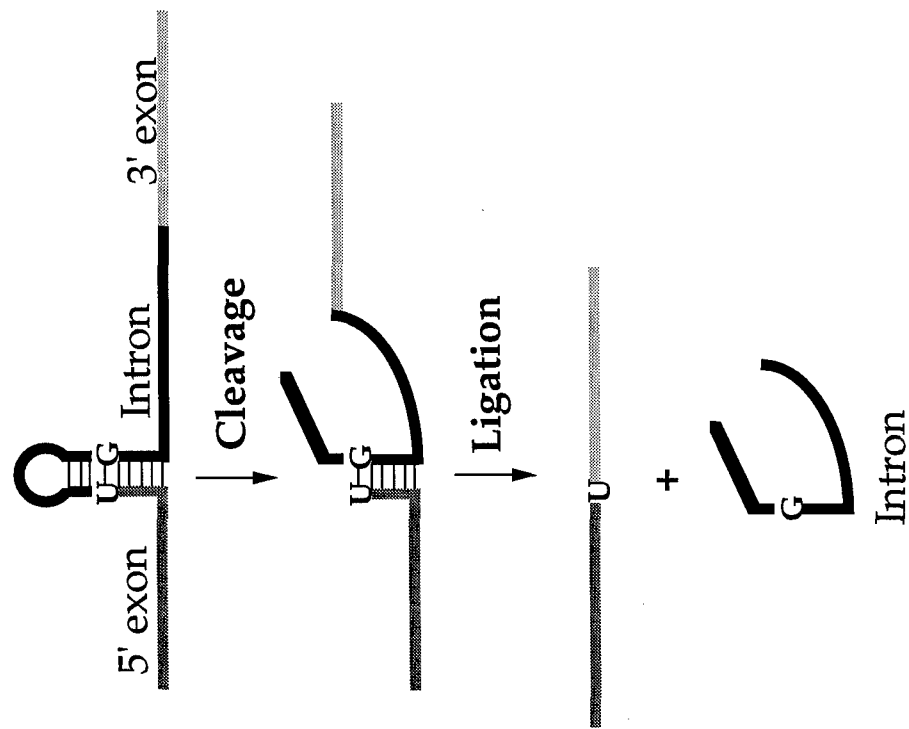
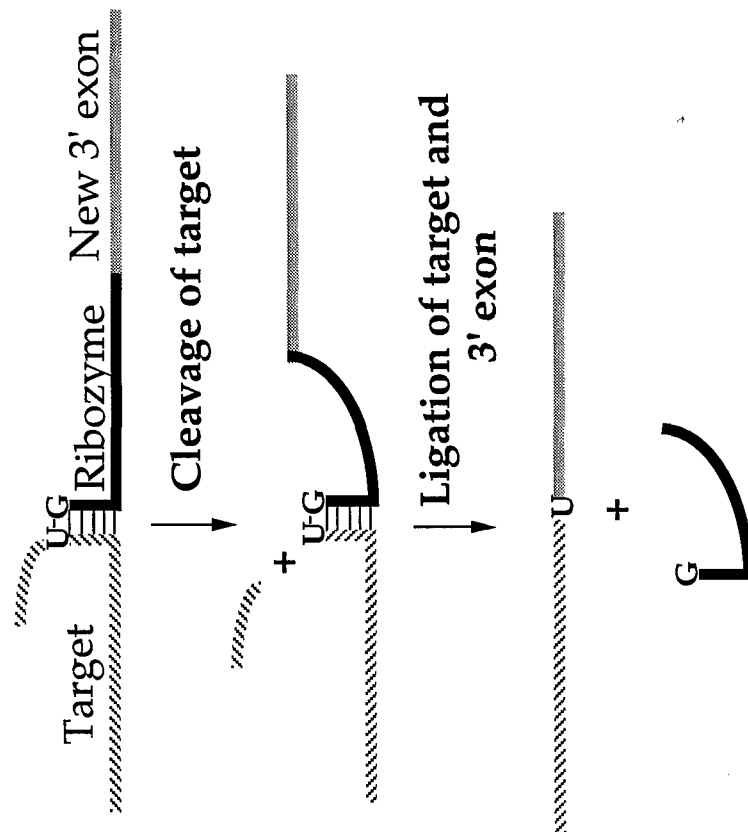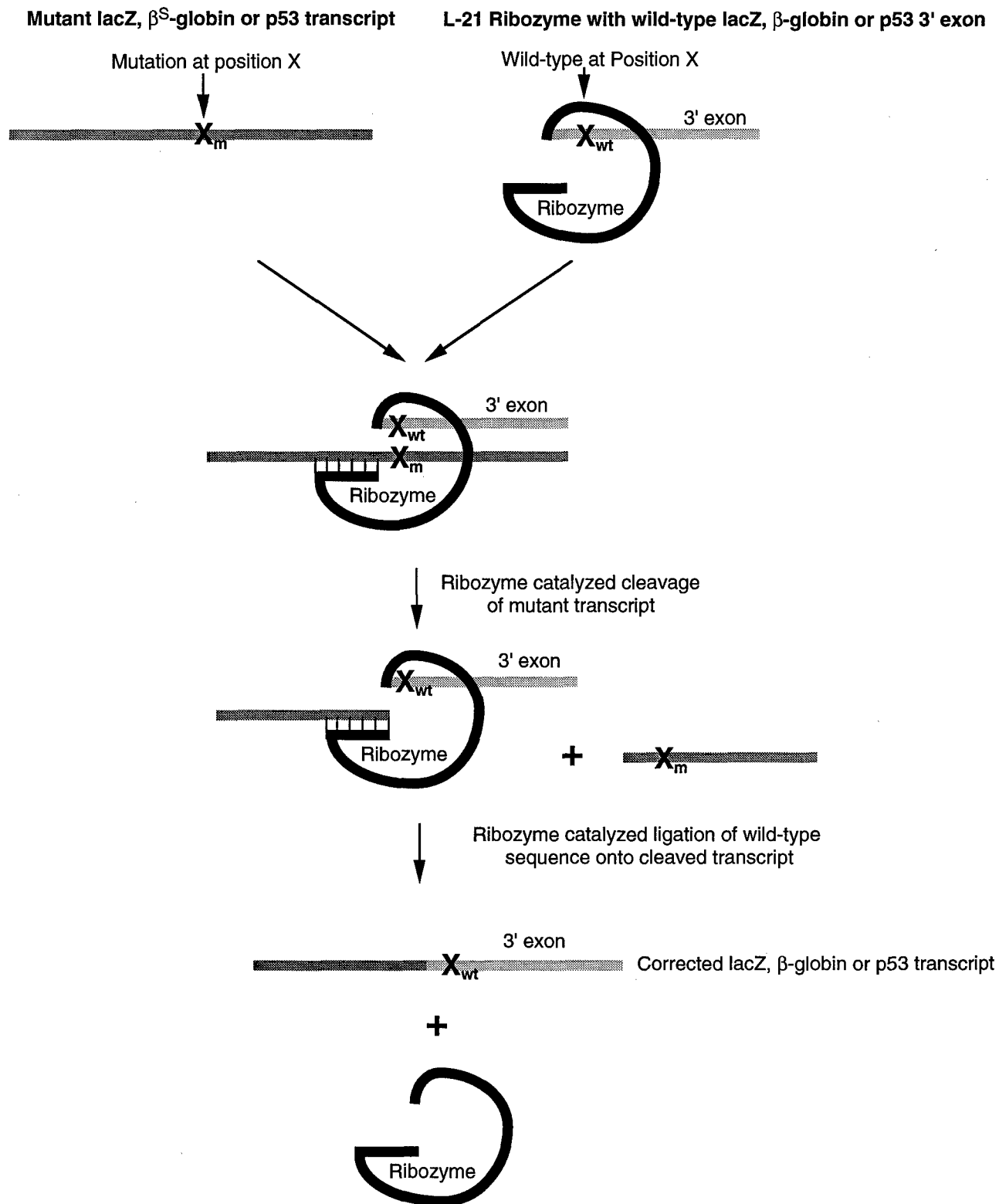B. Targeted trans-splicing of a new 3' exon onto a targeted 5' exon

Figure 2: Targeted Trans-Splicing to Correct Mutant lacZ, Sickle β-globin and p53 Transcripts

Mutant lacZ, β^S-globin or p53 transcript

Mutation at position X

L-21 Ribozyme with wild-type lacZ, β-globin or p53 3' exon

Wild-type at Position X

Ribozyme catalyzed cleavage of mutant transcript

Ribozyme catalyzed ligation of wild-type sequence onto cleaved transcript

Corrected lacZ, β-globin or p53 transcript

plasmid. In contrast, no such amplified product was generated from RNA from mock transfected cells or OST7-1 cells that had been transfected with the inactive ribozyme plasmid. From these results, we concluded that a group I ribozyme can trans-splice its 3' exon onto targeted transcripts in mammalian cells and that this 3' exon provides a convenient molecular tag that allows us to directly follow such RNA catalysis in a therapeutically relevant setting for the first time (Jones et al., 1996).

More recently, we have started to determine if ribozymes can repair clinically relevant transcripts in primary human cells. Toward this end, we recently demonstrated that a trans-splicing ribozyme can amend mutant sickle ß-globin transcripts in erythrocyte precursors isolated from patients with sickle cell disease (Lan et al., 1998). To repair the sickle ß-globin transcripts, the ribozyme has to bind to the mutant RNA at some accessible site upstream of the mutation, cleave the mutant RNA, release the mutation containing product and hold onto the upstream cleavage product, and ligate a 3' exon encoding an anti-sickling version of globin onto the upstream cleavage product to restore the open reading frame for the translation of the globin RNA (Fig. 2). The ribozyme can accomplish such revision with high fidelity (Lan et al., 1998).

1.3. Human gene therapy and RNA repair.

1.3.a. Conventional gene therapy. In this era of molecular medicine, the genetic basis for an increasing number of inherited diseases including many types of cancer is being discovered. Gene therapy represents a new and exciting approach for the treatment of such diseases. Conceptually, gene therapy seems quite simple. To treat a genetic deficiency give a functional copy of the defective gene to the cells of the deficient patient. To accomplish this in practice, most often a viral vector is used to transfer a cDNA copy of the wild type gene, which is usually under the control of a heterologous promoter, to cells harboring a mutant version. If the human genome were a simple warehouse of information, this approach would probably be quite successful. Unfortunately for the gene therapist, our genome appears to be extremely complicated, and expression of the information contained within it is apparently highly regulated. This complexity may severely limit the utility of the simple gene "add back" approach to gene therapy. Regrettably, cDNA versions of genes that are integrated in incorrect locations in the genome and that are expressed from heterologous promoters will almost assuredly not recapitulate the normal expression pattern of endogenous genes. Therefore, unless significant technical advances are made, the gene add back approach may only be useful for the treatment of genetic disorders associated with genes which do not require regulated expression.

Because genes associated with tumorigenesis normally control cell growth and differentiation, their expression will most likely have to be tightly regulated to coordinate cell cycle progression and development. Several observations support this theory. First, deregulated expression of the transcription factor E2F-1 engenders premature entry into S-phase and leads

to p53 dependent apoptosis suggesting that expression of cell cycle proteins must be properly regulated for normal cell replication to proceed (Wu and Levine, 1994; Qin et al., 1994; Shan and Lee, 1994; Kowalik et al., 1995). Furthermore, as pointed out in a recent review (Weinberg, 1992), it has been difficult to restore normal growth to tumor cells harboring a mutant *Rb* gene by retroviral vector mediated-transfer of a wild type cDNA copy of *Rb* to such cells. Although some groups have reported success with such experiments (Huang et al., 1988; Bookstein et al., 1990), other have found that introduction of the *Rb* gene profoundly inhibited cell growth making it difficult to generate enough cells to perform experiments. Finally, it has been demonstrated that overexpression of the wild type *p53* gene aberrantly alters growth and differentiation of normal human keratinocytes (Woodworth et al., 1993). In these experiments, an extra *p53* gene was introduced into primary keratinocytes using a retroviral vector, and this *p53* gene (cDNA version) was expressed from the MoMLV LTR promoter. The cells transduced with the *p53* gene were shown to contain only a 2-4 fold increase in wild type p53 protein levels as compared to cells transduced with control vectors. This modest additional *p53* expression resulted in extremely reduced growth rate, altered differentiation and aberrant expression of genes associated with correct differentiation of keratinocytes (Woodworth et al., 1993). Thus regulated expression of the *p53* gene is important for proper growth and differentiation of primary human cells and incorrect expression can lead to dramatic phenotypic aberrations.

To summarize: a.) cDNA copies of genes introduced into cells do not faithfully recapitulate the expression pattern of endogenous genes.
b.) Dominant and suppressor oncogenes require regulated expression to properly control cell growth and differentiation. Thus, novel experimental approaches that result in regulated expression of oncogenes must be developed to facilitate gene therapy based treatments of cancer.

1.3.b. Ribozyme-mediated repair of mutant transcripts for gene therapy. RNA revision represents an exciting alternative approach to gene therapy that may allow for regulated expression of genes. Trans-splicing ribozymes can be employed to alter RNA messages. With this technique, defective transcripts, which are associated with various genetic diseases, can be emended to encode a wild type version of a gene product (Fig. 2). This approach will mainly be useful for conditions resulting from a defective gene that contains a point mutation or deletion that does not alter the expression pattern of the gene. In those instances, trans-splicing should yield a corrected gene product only at proper times because repair can only occur when the targeted, mutant transcript is present. In addition, RNA repair may be especially appropriate for the treatment of genetic disorders characterized by gene defects that result in the production of deleterious or dominant mutant proteins such as sickle cell anemia and cancers containing dominant mutant oncogenes. Ideally, genetic treatment of these maladies should inhibit the production of the mutant protein and engender the production of the wild

type product. By repairing mutant messages, trans-splicing ribozymes can accomplish both tasks simultaneously.

1.4. p53 loss in human tumors.

1.4.a. The p53 protein. The p53 phosphoprotein was first identified in extracts from simian virus 40 (SV40) induced tumors (Linzer and Levin, 1979; Lane and Crawford, 1979). Subsequently, a variety of *p53* cDNA and genomic clones were shown to be able to immortalize cells in tissue culture and to cooperate with other oncogenes in the transformation of primary cells (Parada et al., 1984). These observations led to the classification of *p53* as an oncogene. However, the cDNA clones of the *p53* gene used in these transformation experiments turned out to encode mutant forms of the p53 protein (Hind et al., 1989). Much information now suggests that the *p53* gene is actually a tumor suppressor gene (Levine et al., 1991). p53 protein levels dramatically increase in response to DNA damage (Maltzman and Czyzyk, 1984; Kastan et al., 1991), and after gamma-irradiation, p53 blocks the progression of the cell cycle in the G1 phase to allow for DNA repair (Kuerbitz et al., 1992). Thus, p53 serves as a check point control in the cell cycle to limit the replication of damaged DNA. To accomplish this task, p53 probably turns on a set of genes which can induce apoptosis in certain cell types or G1 arrest in others (Levine et al., 1994; Fisher, 1994).

1.4.b. Mutation of the *p53* gene in breast cancer. Tumor suppressor genes are often mutated in transformed cells. Thus loss of tumor suppressors appears to be a critical event during neoplastic transformation. In fact about 60% of human cancers have mutations in the *p53* gene suggesting that *p53* mutations may be the most common events in neoplastic transformation (Levine et al., 1991). A variety of mutations can apparently inactivate the p53 protein. Some cells have totally lost the *p53* gene, however most express mutant *p53* transcripts that contain missense point mutations between codons 120 and 290 of the 393 codon long gene (Levine et al., 1991). In breast cancers, the *p53* gene is mutated approximately 40% of the time with most mutations found in exons 5 - 8 of the gene (Lemoine, 1994). For example, the breast carcinoma cell line MDA-MB231 has been shown to contain a single allele of the *p53* gene with a point mutation at codon 280. Here we propose to repair the mutant transcript issuing from this mutant *p53* gene.

2. Body
2.1. Overview

We have made significant progress toward fulfilling several of our specific aims since we received this grant on August 15th, 1997. One accomplishment is that we have determined which regions of the p53 transcript are accessible to trans-splicing ribozymes. Such mapping has been performed upon p53 transcripts generated by in vitro transcription and mutant p53 mRNAs present in total cellular RNA isolated from MDA-MB-231 mammary carcinoma cells.   These results fulfill our first specific aim: to

map mutant p53 transcripts. More recently, we constructed two trans-splicing group I ribozymes that can recognize two of the most accessible regions on the p53 mRNA and have tested the ability of this ribozyme to react with the intended nucleotide on the targeted p53 transcript. Our results demonstrate that such trans-splicing ribozymes can react with p53 transcripts with high fidelity in the test tube as well as in MDA-MB-231 breast cancer cells. These results partially fulfill our specific aims #2 and 4: to create trans-splicing ribozymes that can repair p53 transcripts and test their activity in test tubes and in cells.

2.2 Studies and Results on Objective #1: To determine which regions of mutant *p53* transcripts are accessible to trans-splicing ribozymes (six months).
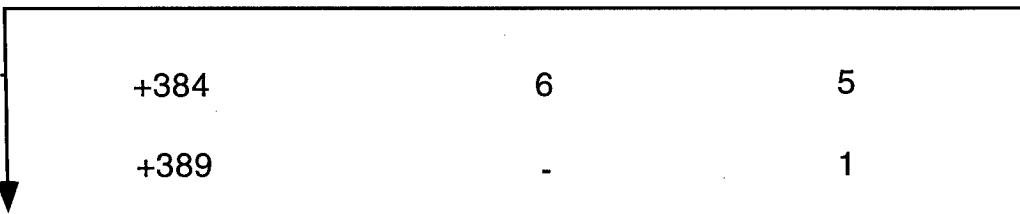
To ascertain which regions of the p53 transcript are accessible to ribozymes, we developed an RNA mapping strategy that is based on a trans-splicing ribozyme library and RNA tagging (Jones et al., 1996). To generate the mapping library, the guide sequence of the *Tetrahymena* group I trans-splicing ribozyme was randomized such that the 5' end of the RNAs in the library began with 5'-GNNNNN-3' where "G" represents guanine and "N" represents equal amounts of the 4 nucleotides. To map the p53 transcript in vitro, the mapping library was incubated under splicing conditions with either p53 transcripts generated by in vitro transcription using T7 RNA polymerase or with p53 transcripts present in total cellular RNA isolated from MDA-MB-231 breast cancer cells. To identify accessible uridine residues, the trans-splicing reaction products were reverse transcribed (RT) and amplified by the polymerase chain reaction (PCR) with primers specific for the ribozyme's 3' exon tag (Jones et al., 1996) and for the 5' end of the p53 target RNA. From this analysis, the uridines at positions 41, 65 and 384 of p53 RNA appeared to be particularly accessible in p53 transcripts regardless of whether the p53 transcripts were generated by in vitro transcription or isolated from MDA-MB-231 mammary carcinoma cells (Fig. 3). These mapping results, taken together with the fact that tumor cells often have mutations downstream of the nucleotide at position 360, encouraged us to focus on developing ribozymes that recognize the uridines present at positions 41 and 65 on the p53 mRNA. Thus, the internal guide sequence on the L-21 trans-splicing ribozyme was changed to 5'-GGAGGG-3' to generate a ribozyme, called Rib41, specific for site 41 and to 5'-GGGUCU-3' to generate a ribozyme, called Rib65, specific for site 65. In addition, inactive versions of these ribozymes, called Rib41d and Rib65d, which lack part of the catalytic core of the enzyme, were generated as controls (Sullenger and Cech, 1994).

2.3 Studies and Results on Objective #2: To construct trans-splicing ribozymes that can repair mutant *p53* transcripts and test them in vitro (six moths).

Rib41 and Rib65 can trans-splice a 3' exon tag onto p53 transcripts in vitro. The trans-splicing ribozymes, Rib41-3'tag and Rib65-3'tag, were incubated under splicing conditions with p53 RNA generated by in vitro transcription or total RNA isolated from MBA-MB-231 cells. To determine if

Figure 3: Mapping Results of the Accessible Uridines on the p53 Transcript

reaction sites (nt)	number of clones	
	in vitro	cellular RNA from MDA-MB-231 cells
+24	49	-
+41	4	4
+65	2	2
+307	-	1
+332	-	1
+340	1	1
+384	6	5
+389	-	1

hot spots of mutations in human cancers

trans-splicing had occurred in any of the RNA samples, RT-PCR analyses were performed with one primer specific for the p53 target RNA and the other primer specific for the 3' exon tag sequence. An amplified fragment of the expected size was generated from samples containing Rib41-3'tag and Rib65-3' Tag and either in vitro transcribed p53 RNA or total RNA isolated from breast cancer cells (unpublished results). No such RT-PCR product was generated from samples that lacked a ribozyme or that contained the inactive versions of the ribozymes. Sequence analysis of the spliced products demonstrated that the ribozyme had reacted with the intended uridine. More recently, we have changed the 3'-exon attached to Rib41 so that it encodes the wild type sequence for the p53 transcript. Using this ribozyme, we have now been able to demonstrate that this ribozyme can repair p53 transcripts, that contain a point mutation at nucleotide 820, in the test tube (unpublished results).

2.4. Studies and Results on Objective #4: To determine if the ribozyme can repair endogenous mutant *p53* transcripts in breast cancer cell lines harboring defective copies of the *p53* gene (nine months).

To determine if Rib41-3'tag could react with mutant p53 transcripts inside human mammary carcinoma cells, the ribozyme was introduced into MBA-MB-231 cells via liposome-mediated transfection. RT/PCR amplification generated a fragment of the expected size (87 base pairs) from the total RNA isolated from the MBA-MB-231 cells that had been transfected with the active ribozyme (Fig. 4). By contrast no such product was generated from RNA samples isolated from cells that were not transfected or were transfected with the inactive ribozyme, Rib41d-3'tag. When Rib41-3'tag was added to the RNA extraction buffer used to isolate total RNA from a sample of mock transfected MBA-MB-231 cells, no amplification product was generated (Fig. 4) suggesting that the observed trans-splicing products were generated inside the transfected cells and not during RNA analysis. From these results we conclude that Rib41 can react with p53 transcripts inside human breast cancer cells. To confirm this result, we subcloned and sequenced a number of these PCR generated cDNAs shown in figure 4. The splice junctions on each of the 6 clones tested were identical and the sequence of a representative clone is shown in figure 5. This sequence analysis demonstrated that the ribozyme had reacted with the uridine at position 41 in the p53 transcript inside of these cells with high fidelity because in all 6 clones sequenced the 3' exon tag had been attached onto the proper uridine.

3. Conclusions

In summary, we have made significant progress toward fulfilling several of our specific aims since we received the funding for this research proposal on August 15th, 1997. We have determined which regions of the p53 transcript are accessible to trans-splicing ribozymes using a novel RNA mapping strategy based upon a library of ribozymes and 3'-exon tagging. Such

Figure 4. Amplification of p53 derived trans-splicing products
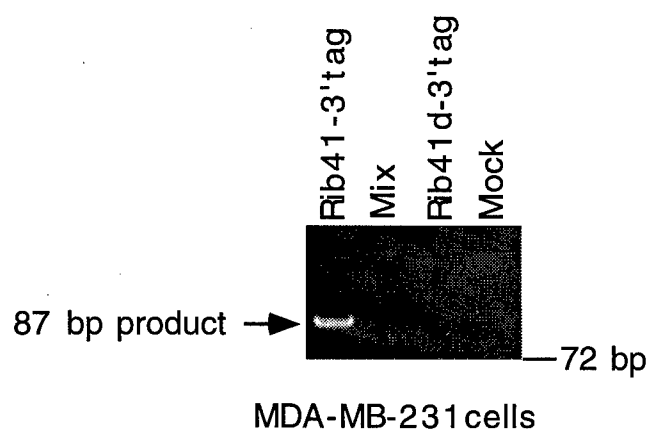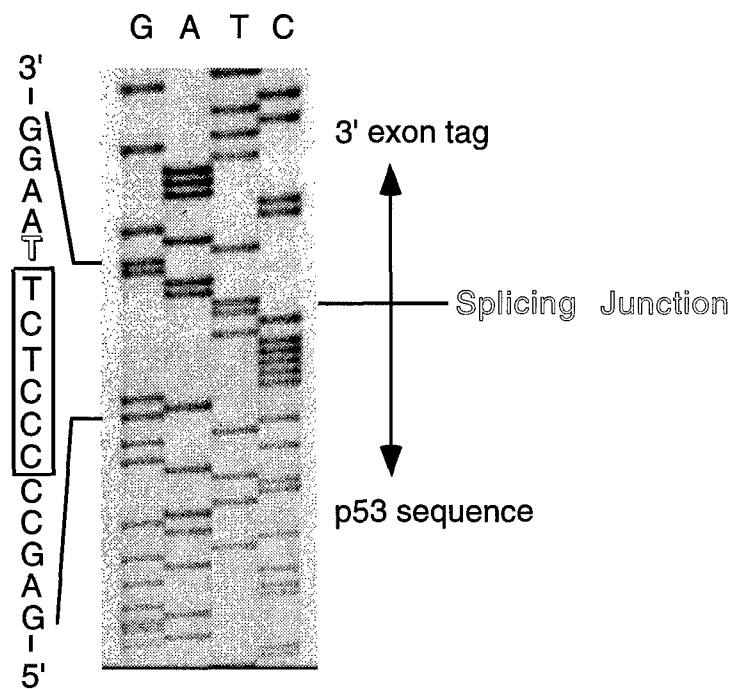

MDA-MB-231 cells

Figure 5. Sequence analysis of trans-spliced reaction products.

MDA-MB-231 cells

mapping has suggested that the nucleotides at positions 41 and 65 on the p53 transcript are particularly accessible to trans-splicing ribozymes. These results fulfill our first specific aim: to map the accessible regions on mutant p53 transcripts. In addition, we constructed two trans-splicing group I ribozymes that can recognize the uridines at positions 41 and 65 on the p53 mRNA and have tested the ability of these ribozymes to react with the intended nucleotide on the targeted p53 transcripts in the test tube and inside the human mammary carcinoma cell line MDA-MB-231. Our results demonstrate that these trans-splicing ribozymes can react with p53 transcripts with high fidelity in the test tube and that Rib41 can react with mutant p53 transcripts in breast cancer cells. These results partially fulfill specific aims #2 and 4: to create trans-splicing ribozymes that can repair p53 transcripts and test their activity in test tubes and in cells. This next year, we plan to determine if Rib65 can react with mutant p53 transcripts in MDA-MB-231 and other breast cancer cells as well as determine if Rib41 can react with mutant p53 transcripts in breast cancer cell lines other than MDA-MB-231. In addition, we will evaluate whether these ribozymes can repair the mutant p53 mRNAs inside these cells and plan to begin to assess what phenotypic effect(s) such repair has upon these cells.

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MCMR-RMI-S   (70-1y)   23 Aug 01

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